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Purification and Properties of a Thermoactive Xylanase from Aspergillus oryzae DSM1863

Thi Tuyen Do and Dinh Thi Quyen

Institute of Biotechnology, Vietnam Academy of Science and Technology,
18 Hoang Quoc Viet Road, Distr. Caugiay, 10600 Hanoi, Vietnam

Abstract: An extracellular xylanase was isolated and purified to homogeneity from crude culture of *Aspergillus oryzae* DSM1863 after 4 days of growth in the mineral medium and its properties were investigated. After a two-step purification scheme involving gel filtration chromatography and ion exchange chromatography, the xylanase was purified 11 times with a yield of 28%. The purified xylanase had a specific activity of 6768 U/mg protein and was a monomeric protein with a molecular mass of 21 kDa determined by SDS-PAGE. Optimum temperature and pH was 60°C and 6, respectively. The enzyme was stable at the temperature range of 37-50°C with a high residual activity of above 62% for 8 h and in the pH range 5-8 with a residual activity of above 84% for 4 h. Metal ions Cu^{2+}, Hg^{+}, Fe^{3+}, and Co^{2+} showed an obvious inhibitory effect on the xylanase activity with a residual activity of 50-72% whereas other tested metal ions inhibited slightly. The enzyme lost 33-46% and 14-28% activity in presence of 10% (v/v) of organic solvents (methanol, ethanol, isopropanol, n-butanol, and acetone) and 2% (w/v) of detergents (Triton X-100 and Tween 20), respectively. SDS completely inhibited the enzyme. The biochemical properties of this enzyme make itself highly valuable for downstream biotechnological applications including feed enzymes.

Keywords *Aspergillus oryzae* DSM1863 · Xylanase · Purification · Characterization

INTRODUCTION

Xylan is a major ingredient of the hemicellulose complex. To hydrolyze xylan, the cooperative action of several enzymes of different functions is necessary. The enzymes involved in the degradation of main polysaccharide chain are endo-1,4-β-D-xylanase (EC 3.2.1.8) randomly cleaving the xylan backbone, β-D-xylosidase (EC3.2.1.37) cleaving xylose monomers from the non-reducing end of xylooligosaccharides. Further enzymes including α-L-arabinofuranosidases (EC 3.2.1.55), α-D-glucuronidases (EC 3.2.1.139), acetylxylan esterases (EC 3.1.1.72), ferulic acid esterases (EC 3.1.1.73) and p-coumaric acid esterases (EC 3.1.1.-) catalyse the removal of side groups of xylans [1].

Endoxylanases are used for pulp industry as it supports the bleaching process, reduce the toxic chemicals (chlorine) used to bleach lignin contained in the paper [2]. Other important applications of xylanases are to make the bread fine and soft and extend the storage time [3]; to purify fruit juice, wine and beer [4]; and to form xylitol glucose used in confectionary industry. In the breeding, xylanase expedites the digestion process of food containing xylan and at the same time helps to reduce the viscosity in the digestive system and follow by many positive effects such as improved food absorption, improved microorganism of the intestine in the advantageous direction, reduced digestion disorder and drier excrement [5, 6].

A variety of microbes, including bacteria, yeast and filamentous fungi, has been reported to produce xylanases, in which the most potent producers are fungi [7]. A number of xylanases have been purified from a wide variety of microbes such as *Bacillus* strains [2] and *Aspergillus* strains [8-12]. However, xylanases are produced mainly by *Aspergillus* and *Trichoderma* sp. on an industrial scale. The present study described purification and characterization of properties of the xylanase produced by *A. oryzae* DSM1863.

Corresponding Author: Dinh Thi Quyen, Institute of Biotechnology, Vietnam Academy of Science and Technology,
18 Hoang Quoc Viet Road, Caugiay District, 10600 Hanoi Vietnam.
Tel: +84-04-37568260, E-mail: quyen@ibt.ac.vn & quyendt2000@yahoo.com

MATERIALS AND METHODS

Chemicals: Birch wood xylan was purchased from Biochemika; 3,5-dinitrosalicylic acid (DNS) from Fluka (Germany). Sephadex G-200 and DEAE-Sephadex A-50 were supplied by Phamarcia Co. (Sweden); SDS from Sigma and Tween 80 from BioBasic Inc. (USA); Triton X-100 from Merck (German). All other chemicals were of analytical grade unless otherwise stated.

Strain Cultivation: The filamentous fungus *Aspergillus oryzae* DSM1863 purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ), was grown in 250 ml Erlenmeyer flasks containing 50 ml of nutrient medium with the following composition (g/l): $NaNO_3$ 2, K_2HPO_4 1, $MgSO_4$ 0.5, KCl 0.5, soybean powder 10, corn cobs 40, pH 7. The inoculated flasks were incubated for 4 days at 30°C on a 200 rpm rotary shaker.

Xylanase Purification: The culture was centrifuged for 10 min at 8000g. Eight ml of the crude enzyme extract (114.3 units) was applied to a Sephadex G-200 column (2.6 x 6 cm) pre-equilibrated with 50 mM potassium phosphate buffer pH 7.5 at a flow rate of 25 ml/h then washed with the same buffer. The eluate was collected with 1 ml per fraction. A highly active xylanase pool of 5 ml through Sephadex G-200 column was further applied to an ion exchange chromatography DEAE-Sephadex A-50 pre-equilibrated with 50 mM Tris HCl buffer pH 8 containing 50 mM NaCl (buffer A), then washed with the same buffer. The protein was eluted with 50 mM Tris HCl buffer pH 8 containing 1000 mM NaCl (buffer B) at a flow rate of 20 ml/h until $OD_{280nm} < 0.01$. The eluate was collected with 1 ml per fraction. The fractions containing high xylanase activity were pooled and used for characterization. All purification steps were carried out at 4°C, unless otherwise specified.

Xylanase Activity Estimation

Xylanase Activity Was Determined Measuring the Increase in Concentration of Reducing Sugars: Formed by enzymatic hydrolysis of birchwood xylan. A reaction mixture of 100 µl of the crude or purified xylanase containing 0.112 µg total protein was incubated with 400 µl of 0.5% (w/v) birchwood xylan in 20 mM potassium phosphate buffer pH 6.5 at 55°C for 5 min. To arrest the reducing sugar released in the reaction mixture, 1.25 ml of DNS was added. The reduced sugars were determined by measuring the absorbance at 540 nm [13]. D-xylose was used as standard. One unit (U) of xylanase activity was defined as the amount of enzyme that released 1 µmol of xylose per min under the standard assay conditions. All measurements were carried out three times and from these values the average value was taken.

SDS-PAGE and Protein Concentration: The homogeneity and molecular mass of xylanase was determined by 12.5% SDS polyacrylamide gel electrophoresis with Biometra equipment [14]. The gels were stained with Coomassie Brilliant Blue R-250. Protein was estimated by the method of Braford with the bovine serum albumin as standard [15].

Temperature and pH Optima: The pH and temperature optima of xylanase were determined by measuring the activity as described above using 20 mM acetate buffer (pH 3-5) and 20 mM phosphate buffer (pH 6-8), and in the temperature range of 37-70°C, respectively.

Temperature and pH Stability: For the determination of temperature and pH stability, purified enzyme (112 ng protein for each reaction) was preincubated at different temperatures 37-70°C, pH 6.5 for 1-8 hours and in pH range 3-8 (20 mM potassium acetate pH 3-5 and 20 mM potassium phosphate pH 6-8) for 4 hours at 30°C, respectively. The residual activity was then determined.

Effect of Metal Ions, Organic Solvents, and Detergents: Purified enzyme (112 ng protein for each reaction) was preincubated in presence of 5 mM of various metal ions (Fe^{3+}; Mg^{2+}; Fe^{2+}; Cu^{2+}; Ca^{2+}; Co^{2+}; Pb^{2+}, Zn^{2+}, Hg^{+}, Ag^{+}; Ni^{+}; K^{+}) and EDTA at 30°C for 5 hours, in presence of 10% (v/v) of various solvents (methanol, ethanol, isopropanol, acetone, n-butanol and ethyl acetate) at 30°C for 1 hour, and in presence of 2% (w/v) of various detergents (SDS; Tween 80 and Triton X-100) at 30°C for 2 hours. The residual activity was then determined.

RESULTS AND DISCUSSION

Purification of *A. Oryzae* DSM1863 Xylanase: The xylanase production by *A. oryzae* DSM1863 in the mineral medium was 114 U/ml (specific activity of 615 U/mg protein) after 4 days of cultivation. The culture supernatant was applied to gel filtration chromatography (Sephadex G-200) and ion exchange chromatography (DEAE-Sephaxdex A-50). Through Sephadex G-200 (Fig. 1A), the xylanase gained a specific activity of 3768 U/mg protein with a purification factor of 6.13 and a yield of 43% (Table 1). The pooled Sephadex G-200 fractions containing high xylanase activity (3215-4659 U/mg) were

Table 1: Purification steps of xylanase from *A. oryzae* DSM1863

Steps	Volume	Activity (U/ml)	Total activity	Protein (mg/ml)	Total protein (mg)	Specific activity (U/mg)	Purification factor	Yield (%)
Crude enzyme	8	114.3	914.4	0.1858	1.4867	615	1	100
Sephadex G-200	5	78.7	393.6	0.0212	0.1058	3768	6.13	43
DEAE-Sephadex	3	84.3	256.4	0.0126	0.0379	6768	11	28

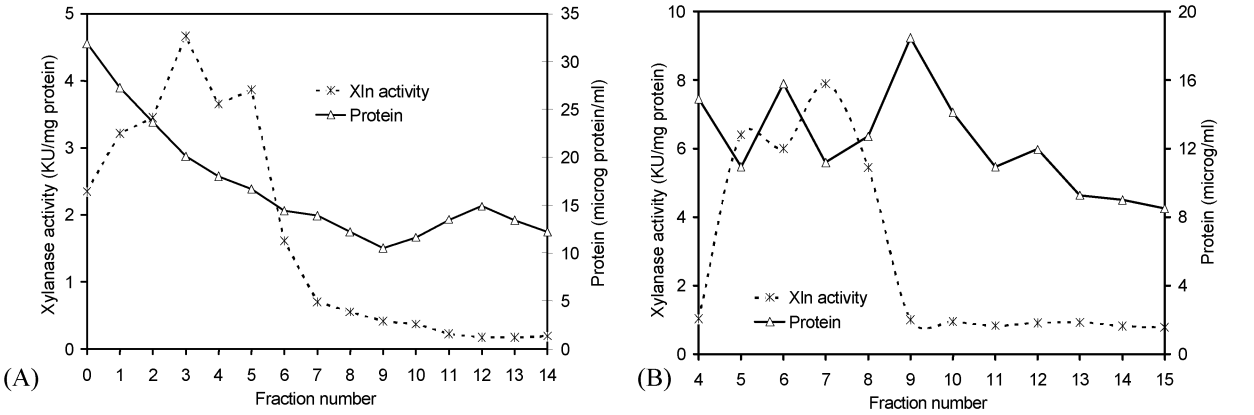

Fig. 1: Gel filtration chromatography Sephadex G-100 (A) and ion exchange chromatography DEAE-Sephadex A-50 (B) of the xylanase from *A. oryzae* DSM1863

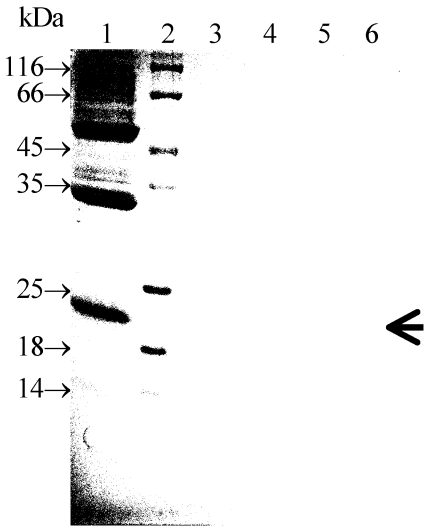

Fig. 2: SDS-PAGE of the purified xylanase from *A. oryzae* DSM1863 through Sephadex G-100 and DEAE-Sephadex A-50; lane 1: the crude enzyme; lane 2: molecular weight marker; lane 3→6: fraction 6→9, respectively.

applied further to the ion exchange chromatography DEAE-Sephadex A-50 (Fig. 1B). The xylanase was purified with a factor of 11 and a yield of 28%. The purified enzyme gained a specific activity of 6768 U/mg (Table 1) and showed a unique protein band on SDS-PAGE (Fig. 2). The molecular weight of this protein was estimated to be 21 kDa.

Other xylanases from *Aspergillus* strains were purified to homogeneity through a similar purification scheme involving ammonium sulfate precipitation, gel filtration chromatography (Sephadex G-200, G-100, G-75), ion exchange chromatography (DEAE-Sephadex A-50, DEAE Sepharose), and affinity chromatography Phenyl Sepharose 6 Fast Flows [9-12]. Purified xylanases from *Aspergillus* strains had various molecular weights from 21 to 35 kDa on SDS-PAGE: 21-22 kDa (*A. sydowii* SBS 45 [12], *A. giganteus* [9], *A.* cf. *niger* [10], *Aspergillus* sp. FP-470 [16]), 24 kDa (*A. giganteus* [9]), 34-35 kDa (*A. nidulans* [8], *A. ficuum* AF-98 [11]), and 43 kDa (*A. sydowii* SBS 45 [12]). The xylanase from *A. oryzae* DSM 1863 had also molecular weight of 21 kDa like it from *A. niger*, *A. sydowii*, and *A. giganteus*. Purification factor was 33 for xylanase from *A. ficuum* AF-98 [11], and 77-93 for xylanases from *A. sydowii* SBS 45 [12], higher than that for xylanase from *A. oryzae* DSM 1863 (11).

Temperature Optimum and Stability: The xylanase from *A. oryzae* DSM1863 had an optimum temperature of 60°C (Fig. 3A) and showed high activity (≥82%) at a large temperature range of 40-70°C in comparison to the maximum activity. The enzyme was stable at 37-50°C for 2 h with a residual activity of ≥75% (Fig. 3B) but the xylanase lost one half of activity when it was treated at 60-70°C just for 1-2 h. Most xylanases from other *Aspergillus* strains had a similar optimum temperature

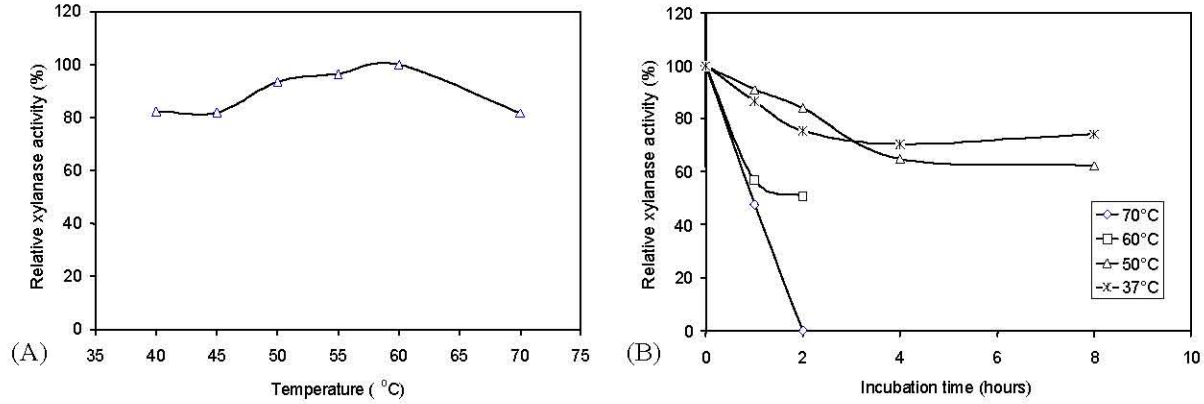

Fig. 3: Temperature optimum (A) an stability (B)

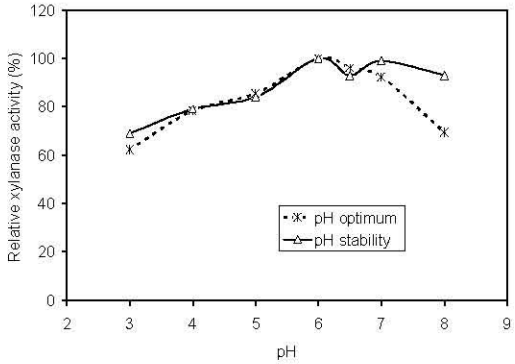

Fig. 4: pH optimum and stability

ranged from 45°C to 60°C. The optimum temperature for *Aspergillus* xylanses was 60°C (*A. oryzae* [17]), 55°C (*A.* cf. *niger* BCC14405 [10], and *A. nidulans* [8]), 50°C (*A. giganteus* [9], *A. sydowii* SBS 45 [12]), and 45°C (*A. ficuum* AF-98 [11]).

pH Optimum and Stability: The xylanase from *A. oryzae* DSM1863 had an optimum pH 6 (Fig. 4) and worked well in a pH range 5-7 with activity of ≥85% in comparison to the maximum activity. The enzyme was stable in a pH range 5-8 for 4 hours with a residual activity of ≥84% (Fig. 4). This finding was coincident with most xylanases from *Aspergillus* strains which showed an optimum pH around 6.0 in acid range: pH 6.0-6.5 (*A. giganteus* [9], *A. nidulans* [8]), pH 5 (*A. oryzae* [17], *A.* cf. *niger* BCC14405 [10], *A. ficuum* AF-98 [11]). In contrast, pH optimum for two xylanases from *A. sydowii* SBS 45 was in alkaline range (pH 10) [12].

Effect of Metal Ions on Xylanase Activity: The addition of Ag^{+} and Pb^{2+} to the final concentration of 5 mM showed no effect on the xylanase activity whereas the addition of other metal ions or EDTA showed an obvious inhibition effect on the xylanase activity. The addition of 5 mM of K^{+}, Zn^{2+}, Ni^{2+}, EDTA Mg^{2+}, Fe^{2+}, and Ca^{2+} inhibited the xylanase slightly with an activity lost of 9-25%, whereas the addition of 5 mM of Cu^{2+}, Hg^{+}, Fe^{3+}, and Co^{2+} decreased the xylanase by 28-50% (Fig. 5A). Fialho and Carmona (2004) reported that the addition of 2 and 10 mM of Mg^{2+}, Ca^{2+}, Zn^{2+}, and Ba^{2+} did not show any effect or slight inhibition on the activity of *A. giganteus* xylanases whereas ions Hg^{2+}, Cu^{2+} strongly inhibited xylanases with an activity lost of 50-100%. Most of the inorganic salts tested (10 mM of Co^{2+}, Zn^{2+}, Ca^{2+}, Na^{+}, Mg^{2+}, Mn^{2+}) showed a slight increase in *A.* cf. *niger* BCC14405 xylanase activity up to 30%. However, 10 mM Cu^{2+}, EDTA, or Fe^{2+} lead to a 40-50% reduction in enzyme activity [10]. In contrast to the xylanase from *A. oryzae* DSM1863, the additives Al^{3+}, Ba^{2+}, Ca^{2+}, Na^{+} and Zn^{2+} at the concentration of 10 mM increased the activity of xylanases from *A. sydowii* SBS 45 [12]. *A. ficuum* AF-98 xylanase activity was increased to 115.8% when enzyme fluid was incubated with metal ion Cu^{2+}, strongly inhibited and decreased to 52.8% and 89% by ions Hg^{2+} and Pb^{2+}, respectively [11].

Effect of Organic Solvents and Detergents: The addition of tested organic solvents at the final concentration of 10% lead to reduction of 34-46% in the xylanase activity (Fig. 5B). The xylanase activity without treatment was fixed as 100%. The addition of 2% (w/v) Triton X-100 and Tween 80 decreased slightly the enzyme activity by 14% and 28%, respectively (Fig. 5B). But, the addition of 2% (w/v) SDS completely inhibited the enzyme activity. This result was coincident with the report that *A. giganteus* xylanase lost also 100% activity when it was treated with SDS [9].

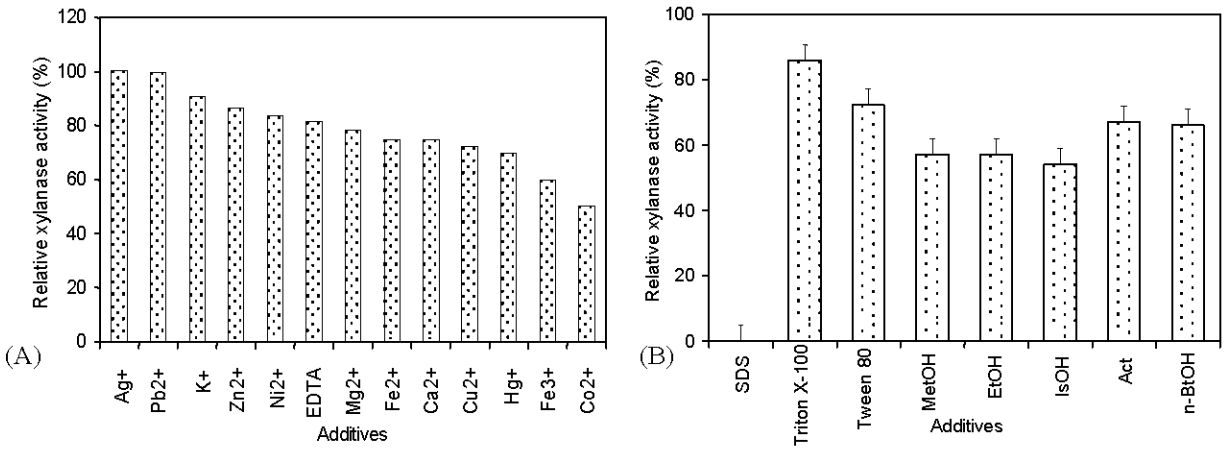

Fig. 5: Effect of metal ions (A), detergents and organic solvents (B) on xylanase activity

CONCLUSION

A xylanase was purified from the culture supernatant of *Aspergillus oryzae* DSM1863 11 fold to homogeneity throughout Sephadex G-100 gel filtration chromatography and DEAE-Sephadex A-50 ion exchange chromatography. The purified xylanase had a specific activity of 6768 U/mg protein and a monomeric protein with a molecular mass of 21 kDa as determined by SDS-PAGE. The enzyme showed temperature optimum of 60°C and pH optimum of 6. The purified xylanase was stable in the temperature range 37-50°C and pH range 5-8. All the additives tested in this study including metal ions, organic solvents and detergents showed a slightly to moderately inhibitory effect on the xylanase activity, only SDS completely inhibited the xylanase from *A. oryzae* DSM1863. The enzyme has potential applications in feed enzyme industry.

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